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Differences in Excretion and Efficiency of the Aerobactin and Enterochelin Siderophores in a Bovine Pathogenic Strain of *Escherichia coli*

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Secretion of aerobactin is thought to play an important part in the virulence of invasive *Escherichia coli* also capable of synthesizing enterochelin. Why, despite its markedly lower affinity for iron than that of enterochelin, aerobactin proves to be the predominant active siderophore for bacterial growth in transferrin was investigated. We studied the action of two iron chelators, 2,2′-dipyridyl and transferrin, in expression of the aerobactin and enterochelin genes. Specifically, we describe the sequential localization of the two siderophores in the cell compartments during bacterial growth under different iron limitation conditions. Our results demonstrated that, whatever the exogenous iron-chelating agent used, aerobactin was rapidly excreted, whereas enterochelin accumulated early in periplasm before its very belated release into the external medium. This work also showed that the advantage of aerobactin over enterochelin in competition with transferrin was not due to (i) lack of enterochelin activity, (ii) a cell-bound aerobactin-dependent mechanism, (iii) antagonism between the two siderophores, and probably (iv) genetic preferential induction of aerobactin. We propose that the superiority of aerobactin in competing with transferrin for iron(III) was a consequence of its more rapid excretion with respect to enterochelin. In contrast to transferrin, 2,2′-dipyridyl induced a greater efficiency of enterochelin, possibly by a more permanent function as iron-binding compounds in the bacterial envelope. In summary, unlike aerobactin, enterochelin appears to be a weakly secreted high-affinity iron ligand.

Iron is a crucial element for bacterial growth and virulence. Although the total iron concentration in mammalian body fluids is high (20 μM) (12), almost all of the iron is tightly bound with specialized iron-binding glycoproteins, such as transferrin in serum and lactoferrin in secretions. This results in a free ionic iron concentration of 10^{-12} to 10^{-10} M (20), far below the bacterial requirements (50 nM to 4 μM). To obtain protein-bound iron, bacteria possess specific high-affinity systems that generally consist of low-molecular-weight iron(III)-chelating compounds called siderophores (22) and their corresponding membrane receptor proteins necessary for siderophore binding and uptake (23). In *Escherichia coli*, two siderophores have been described: the common phenolate-type enterochelin (also called enterobactin) and the hydroxamate-type aerobactin. The latter can be either plasmid or chromosome encoded (3, 26). In contrast to enterochelin, aerobactin is considered an important virulence component in extraintestinal colibacillosis (27, 29). This may be because aerobactin possesses features which result in more effective provision of iron from transferrin and lactoferrin for bacterial growth (19, 28). However, because the enormous thermodynamic advantages in binding iron that enterochelin has over aerobactin and transferrin (22), why aerobactin provides superior activity to remove iron from transferrin is not clear. There are contradictory reports in the literature pertaining to the chemical class of siderophores that are preferentially produced by pathogenic *E. coli* in response to the severity of iron stress (15, 28).

The present study was undertaken to understand how plasmid-mediated aerobactin is able to confer a significant selective advantage for growth in transferrin upon a bovine extraintestinal *E. coli* strain already capable of synthesizing enterochelin. We concluded that the superiority of aerobactin in competing with transferrin for iron is a result of extensive retardation of enterochelin excretion compared with that of aerobactin.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. Wild-type *E. coli* A36 (O8:K87:H9) is a pathogenic isolate collected from diarrheic calf feces in France in 1979. The O:K:H serotyping was characterized by Ida Ørskov at the International *Escherichia* and *Klebsiella* Center, Copenhagen, Denmark. The pathogenic characteristics of this strain have been described previously (6). Strain A36 can utilized raffinose, sucrose, dulcitol, and sorbose as sole sources of carbon and energy for growth. *E. coli* A36 contains three plasmids, namely, pA36-1, pA36-2, and pA36-3, about 121, 98, and 10 kilobases in size, respectively. Plasmid-curing experiments were carried out with ethidium bromide. Conjugative transfer of plasmids was performed on a 0.45-μm (pore diameter) filter membrane; two plasmid-free strains of *E. coli* K-12, DB6433 and IR20/111N, were used as recipients.

Plasmid pA36-1 codes for synthesis of a functional aerobactin-mediated iron uptake system. This was revealed by the fact that pA36-1-carrying strains produced aerobactin, as assessed by a cross-feeding test using *E. coli* mutant LG1522 ($FepA^-$ Aer^- Iut^+) as the indicator strain, and were sensitive to cloacin DF13, which binds to the ferric-aerobactin receptor (2, 14). Plasmid pA36-2 codes for resistance to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline, and sulfamides. Plasmid pA36-3 specifies synthesis of a colicin other than type V.

Media and culture conditions. Strains were routinely cultivated in LB medium (21), MacConkey agar medium (Difco Laboratories, Detroit, Mich.), or M9-CA medium, which is an M9 minimal-salts medium (21) supplemented with 0.2%

TABLE 1. *E. coli* strains used

Strain	Relevant characteristics (plasmid-determined phenotype)[a]	Reference
A36	Pathogenic isolate carrying plasmids pA36-1 (Aer$^+$ Iut$^+$), pA36-2, and pA36-3	This work
A36-749	A36 lacking plasmids pA36-2 and pA36-3	This work
A36-749/11	A36-749 Iut$^-$	This work
A36-749/130	A36-749 lacking plasmid pA36-1	This work
K-12		
KH576	*rpsL nalA* carrying plasmid pColV-K30 (Aer$^+$ Iut$^+$)	27
LG1522	*ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi* carrying plasmid pColV-K30 (Aer$^-$ Iut$^+$)	7
AB2847	*aroB thi tsx malT* Ent$^-$ FepA$^+$	5
AB2847N	AB2847 *nalA*	This work
H646	AB2847 *rpsL cir* carrying plasmid pColV-K311 (Aer$^+$ Iut$^+$)	5
IR20/111	AB2847 *cir fepA fhuA*	17
IR20/111N	IR20/111 *nalA*	This work
DB6433	F$^-$ Δ(*lac pro*) *argE*(Am) *metB nalA rpoB supF* Ent$^+$ FepA$^+$	10
XA/CV30	DB6433 carrying plasmid pColV-K30 (Aer$^+$ Iut$^+$)	This work
XA36/08	DB6433 carrying plasmid pA36-3	This work
XA36/42	XA36/08 carrying plasmid pA36-1 (Aer$^+$ Iut$^+$)	This work
IRA36	IR20/111N carrying plasmid pA36-1 (Aer$^+$ Iut$^+$)	This work
IRA36/80	IR20/111N carrying plasmid pA36-1 (Aer$^-$ Iut$^+$)	This work
IRA36/11	IR20/111N carrying plasmid pA36-1 (Aer$^+$ Iut$^-$)	This work

[a] Ent, enterochelin; FepA, ferric-enterochelin receptor; Aer, aerobactin; Iut, ferric-aerobactin receptor; + and − indicate synthesis and no synthesis, respectively.

glucose, 50 μg of thiamine per ml, and 0.5% Casamino Acids (Difco). The amount of iron present as traces in M9-CA medium was about 2.7 μM. For growing *aroB* strains, the M9-CA medium was supplemented with 100 μg of tryptophan per ml, 100 μg of tyrosine per ml, 100 μg of phenylalanine per ml, 0.2 mM *para*-aminobenzoic acid, 0.2 mM *para*-hydroxybenzoic acid, and, if needed, 1 mM sodium citrate. Antibiotics were used at the following final concentrations: ampicillin, 50 μg/ml; streptomycin, 30 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 34 μg/ml; tetracycline, 10 μg/ml; nalidixic acid, 40 to 100 μg/ml; rifampin, 100 μg/ml. Iron-limited cultures were obtained by adding either human transferrin (Sigma Chemical Co., St. Louis, Mo.) or 2,2′-dipyridyl (E. Merck AG, Darmstadt, Federal Republic of Germany). Iron-sufficient cultures were obtained by adding $FeCl_3 \cdot 6H_2O$. All liquid cultures were grown at 37°C and aerated by shaking.

Human transferrin preparation. Human serum transferrin (98% pure; Sigma) was dissolved in 40 mM Tris hydrochloride buffer, pH 7.4, containing 20 mM $NaHCO_3$ to provide the bicarbonate ions required for iron binding, and sterilized by filtration. After addition of transferrin to the culture medium and before growth experiments, iron was allowed to bind to the transferrin during 30 min of incubation at 37°C.

Bacterial growth. M9-CA medium containing antibiotics or iron chelators, if required, was inoculated from stationary LB cultures. Cultures were incubated overnight and subcultured to fresh medium at a density of approximately 10^6 cells per ml. The growth rate and maximal growth were estimated by periodic measurements of culture turbidity at 610 nm.

Plasmid content analysis. Two plasmid isolation procedures were used. The first method, used to detect large (>200 kilobases) plasmids in wild-type strains, was a modification of the Eckardt procedure (13) described by Rosenberg et al. (24). The second method used was that of Kado and Liu (18).

Chemical tests for siderophore production. Assays for the presence of a conventional extracellular siderophore were performed on filtered supernatant fluids from overnight M9-CA cultures. Production of phenolate compounds was determined at 510 nm by the colorimetric Arnow test (1), with 2,3-dihydroxybenzoic acid as the standard.

Production of hydroxamate compounds was determined by the Csàky assay (9), except that 1 ml of culture supernatant was hydrolyzed with 1 ml of 3 M H_2SO_4 by being autoclaved at 120°C for 4 h and then neutralized by addition of 1.55 ml of a solution of 35% anhydrous sodium acetate. After complete pink color development of the final mixture, the A_{526} was measured. The concentration of hydroxylamine-nitrogen groups was estimated by using a standard curve prepared from known quantities of hydroxylamine hydrochloride autoclaved at 120°C for 4 h in 3 M H_2SO_4. H646 and XA/CV30 were used as positive control strains for aerobactin production. One micromole of enterochelin corresponds to 3 μmol of 2,3-dihydroxybenzoic acid, and 1 μmol of aerobactin corresponds to 2 μmol of hydroxylamine hydrochloride.

Biological assay for aerobactin production. Production of aerobactin was detected by a cross-feeding test as described by Carbonetti and Williams (7). We used *E. coli* LG1522 as the indicator. This mutant is impaired in uptake of enterochelin and synthesis of aerobactin. The receptor of the ferric-aerobactin complex was not affected. Thus, this strain can grow in iron-restricted conditions if provided with aerobactin but not enterochelin.

Biological assay for ferric-aerobactin receptor activity. The presence of the aerobactin outer membrane receptor was assayed by the cloacin sensitivity test (7). Cloacin DF13 was prepared by adding mitomycin C to a growing nutrient broth culture of *Enterobacter cloacae* S458 as described by De Graaf et al. (11), except that the crude bacteriocin preparation was neither concentrated nor purified. XA/CV30 and H646 were used as sensitive control strains, and DB6433 and IR20/111N were used as resistant control strains. Cloacin-resistant mutants were isolated from overlays of nutrient agar plates containing 0.2 mM 2,2′-dipyridyl, 1 mM sodium citrate, and 1 ml of the undiluted crude cloacin DF13 preparation.

Cellular localization of aerobactin and enterochelin during bacterial growth. Fractionation of extracellular, periplasmic,

and cytoplasmic siderophores in *E. coli* cells was performed by the method of Yanagida et al. (30). Strains were grown in M9-CA medium containing either 3.12 μM transferrin or 200 μM 2,2'-dipyridyl. At hourly intervals during bacterial growth, samples (40 ml) of cultures were harvested by centrifugation and washed twice with 10 mM Tris hydrochloride buffer (pH. 7.5) containing 25% sucrose. The washed cells were suspended in the same buffer containing 25% sucrose and 1 mM EDTA, and the suspension was shaken for 10 min at room temperature. After centrifugation at 7,000 × *g* for 10 min, the cells were quickly and vigorously suspended in 0.4 ml of ice-cold water. The suspension was further shaken for 10 min at 4°C and centrifuged at 9,000 × *g* for 10 min. The precipitated cells were suspended in 0.4 ml of 10 mM Tris hydrochloride buffer (pH. 7.5) and disrupted by sonication. The extracellular siderophore fraction was the activity in the filtered culture supernatant. The periplasmic fraction was the activity found in the supernatant after treatment with cold water. The cytoplasmic fraction was the activity found in the supernatant after sonication. The aerobactin and enterochelin activities were estimated by the abilities of these different fractions to support the growth of IRA36/80 and AB2847N cells in M9-CA agar medium (10^7 cells per plate) containing 50 μg of nalidixic acid per ml and either 200 μM 2,2'-dipyridyl or 3.12 μM transferrin. Strains IRA36/80 and AB2847N were used as the aerobactin and enterochelin indicators, respectively. Cross-feeding assays were tested by filling large wells (8-mm diameter) with 200 μl of hourly sampling fractions. After overnight incubation, positive cross-feeding was observed as a halo of growth of the lawn strain around the well.

RESULTS

Effects of iron chelators on siderophore excretion. The effects of various concentrations of transferrin and 2,2'-dipyridyl on excreted siderophore production were measured in a supernatant fraction of overnight *E. coli* A36-749 cultures. Aerobactin production per cell yield increased linearly with 2,2'-dipyridyl concentrations between 60 and 200 μM and with transferrin concentrations between 3.75 and 10 μM (Fig. 1). That of enterochelin remained approximately constant when up to 200 μM 2,2'-dipyridyl was used but increased linearly with increasing transferrin concentrations up to 10 μM. Thus, only enterochelin excretion was not subjected to increase of the external 2,2'-dipyridyl concentration. By contrast, excretion of both siderophores was sensitive to external transferrin concentration.

Excretion of enterochelin and aerobactin during the growth of A36-749 cells was initially estimated by the chemical tests of Arnow and Csàky, respectively (data not shown). In the presence of 2,2'-dipyridyl, hydroxamate secretion started in the early exponential phase, paralleled the growth curve, and continued during the stationary phase. On the other hand, phenolate release was only detected in the stationary growth phase. With transferrin, both compounds were evidenced mainly during stationary growth. Because of the detection limits of the Arnow and Csàky methods, we were not sure whether any siderophore was secreted by cells growing exponentially in transferrin. Consequently, we used a 15 to 20 times more sensitive cross-feeding assay for detection of siderophores under transferrin induction. This bioassay followed the procedure described in Materials and Methods relevant to cellular localization of siderophores. A36-749 cells growing in transferrin excreted enterochelin in the late logarithmic and stationary phases and excreted aerobactin before the early logarithmic phase after 3 h of growth (Fig. 2). Enterochelin was secreted 5 h later than aerobactin, indicating preferential excretion of the latter siderophore by cells capable of synthesizing the two siderophores. On the other hand, strain A36-749/130, with enterochelin alone, excreted its siderophore in the early logarithmic phase after an initial nonlogarithmic growth time of 8 h (Fig. 2). External 2,3-dihydroxybenzoyl group levels increased eightfold per cell division during exponential growth of A36-749/130 cells (Fig. 2). Strains A36-749 and A36-749/130 released enterochelin at the same time (Fig. 2), suggesting that the enterochelin synthesis-encoding genes under transferrin induction were expressed similarly in these two isogenic strains.

Effects of iron chelators on cell growth. After 4 or 5 h of nonlogarithmic growth, strain A36-749 grew exponentially in 3.12 μM transferrin, with a growth rate of 1.09 h^{-1} (Fig. 2). By contrast, the growth of strain A36-749/130 was highly affected. However, after an initial nonlogarithmic growth time of 8 h, the latter strain was able to overcome the transferrin-induced bacteriostasis with a growth rate of 0.5 h^{-1} (Fig. 2). This was not due to inactivation of transferrin with time. Indeed, in contrast to IRA36, a strain that synthesized aerobactin only, mutants deficient in both iron uptake systems (strains IRA36/80 and IRA36/11) were permanently inhibited (data not shown). The growth of A36-749/11, an enterochelin-producing strain only impaired in aerobactin receptor synthesis, was also affected by transfer-

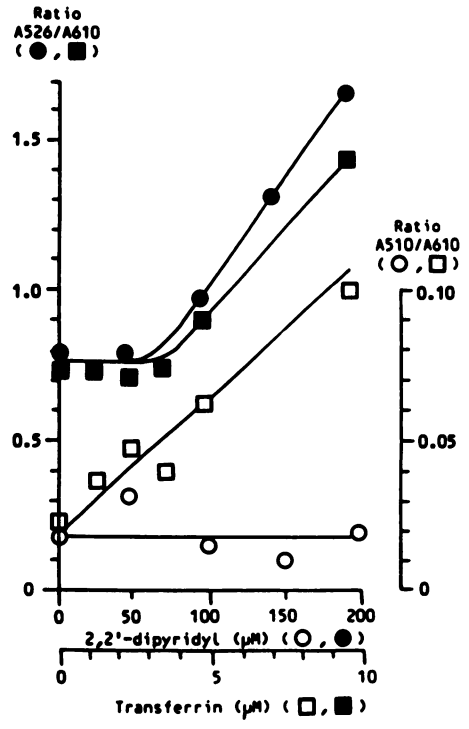

FIG. 1. Excretion of aerobactin (closed symbols) and enterochelin (open symbols) in the presence of various concentrations of 2,2'-dipyridyl (round symbols) and transferrin (square symbols). Excretion of both siderophores was estimated from supernatants of stationary M9-CA cultures of A36-749 cells according to the Csàky and Arnow tests. Excretion of hydroxamate and phenolate compounds was determined by measuring the A_{526} and A_{510}, respectively. The measure of the A526/A610 and A510/A610 ratios reflects the production of aerobactin and enterochelin per cell yield, which was estimated by measuring optical density at 610 nm.

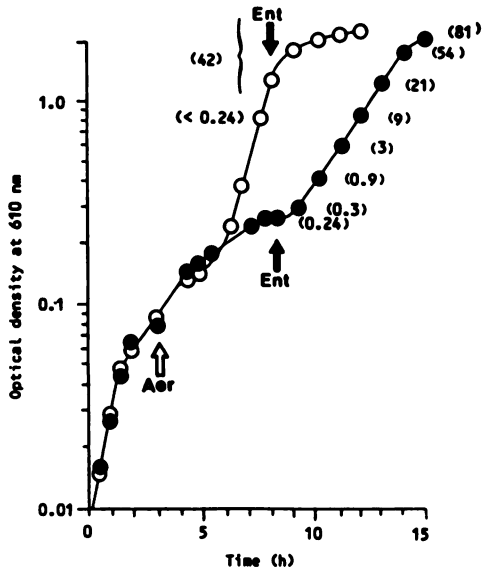

FIG. 2 Excretion of the enterochelin and aerobactin siderophores during growth of the Aer$^+$ Ent$^+$ strain A36-749 (○) and the Aer$^-$ Ent$^+$ strain A36-749/130 (●) in M9-CA medium containing 3.12 μM transferrin. Excretion of aerobactin and enterochelin was estimated as described in the section of Materials and Methods on cellular localization of siderophores. Bacterial growth was monitored by periodic measurements of optical density at 610 nm. The open and closed arrows designate the culture supernatant sample in which detectable aerobactin (Aer) and enterochelin (Ent), respectively, were first evidenced. The values given in parentheses indicate the concentration (micromolar) of 2,3-dihydroxybenzoyl (DHB) groups in each sample. This was determined by measuring the diameter of the halo of growth of the lawn strain around the well. The relationship between DHB group concentration and growth diameter was estimated by using a standard curve prepared from twofold dilutions of a solution containing phenolate compounds (stationary culture supernatant from strain A36-749/130) which were quantified by the method of Arnow.

rin (data not shown). Moreover, at 5 μM transferrin, the maximal A36-749 and A36-749/130 cell yields were decreased by 20 and 80%, respectively (Fig. 3). All of these findings confirmed that aerobactin is the predominant active siderophore for bacterial growth in transferrin. To examine whether aerobactin activity requires surface contact between bacterial cells and transferrin molecules, we tested the growth capacity of enterochelin-deficient mutants that do or do not produce aerobactin (strains IRA36 and IR20/111N, respectively) when separated from transferrin by a dialysis membrane (data not shown). No growth was observed only in strain IR20/111N. Therefore, removal of transferrin-chelated iron by aerobactin does not require surface contact between bacterial cells and transferrin molecules.

The type of chelator used to cause iron restriction influenced growth parameters. At 100 μM 2,2'-dipyridyl, the maximal growth of strain A36-749/130 was relatively unaffected, whereas that of strain A36-749 was inhibited by almost 50% (Fig. 3). This differential effect of 2,2'-dipyridyl may be explained by a more energy-expensive way of assimilating iron because of simultaneous involvement of both iron uptake systems. The differences between enterochelin-producing strains with and without aerobactin were in their lag phases but not their growth rates (Fig. 4). These data suggest that, although enterochelin alone was able to supply sufficient iron, both siderophores operated in concert for growth in 2,2'-dipyridyl.

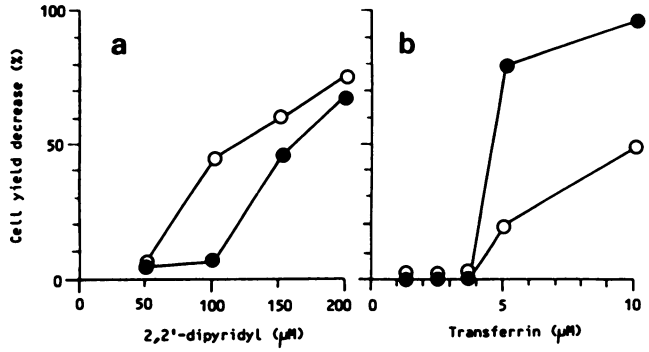

FIG. 3. Effect of increasing 2,2'-dipyridyl (a) and transferrin (b) concentrations on maximal growth of the Aer$^+$ Ent$^+$ strain A36-749 (○) and the Aer$^-$ Ent$^+$ strain A36-749/130 (●). The strains were grown overnight in M9-CA medium. Maximal growth was estimated by measurement of optical density at 610 nm. The percentage of cell yield decrease was calculated from maximal growth values obtained with and without iron chelators.

Cellular localization of aerobactin and enterochelin during bacterial growth. A36-749 cells excreted aerobactin after 3 h of growth in transferrin and after 2 h of growth in 2,2'-dipyridyl (Table 2). Aerobactin was always detected only 1 and 2 h later in periplasm and cytoplasm, respectively. Therefore, aerobactin was secreted immediately after its synthesis, whatever the iron-chelating agent used. By contrast, enterochelin was not excreted for up to 7 h of growth under the iron limitation conditions imposed by either transferrin or 2,2'-dipyridyl (Table 2). However, the phenolate compound was found in the periplasmic space of A36-749 cells after 3 h of growth in transferrin and after 2 h of growth in 2,2'-dipyridyl (Table 2). This indicated that enterochelin always accumulated early in the periplasm after its synthesis and before its belated release into the external medium. Accordingly, although the two siderophores were first detected at the same time, whatever the source of iron deficiency, aerobactin was always more rapidly secreted than enterochelin.

DISCUSSION

This study, in good agreement with other (19, 28), demonstrated that aerobactin was more effective than enteroche-

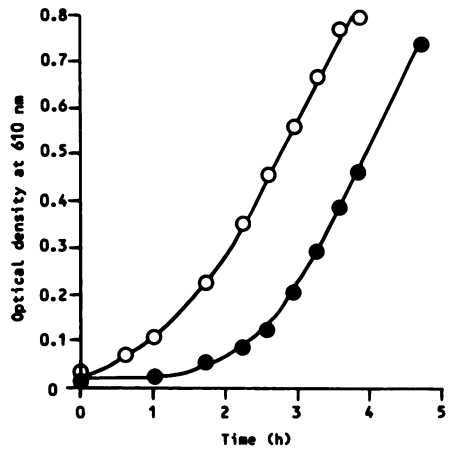

FIG. 4. Growth of the Aer$^+$ Ent$^+$ strain A36-749 (○) and the Aer$^-$ Ent$^+$ strain A36-749/130 (●) in the presence of 2,2'-dipyridyl. The strains were grown in M9-CA medium containing 0.2 mM 2,2'-dipyridyl. Bacterial growth was monitored by periodic measurements of optical density at 610 nm.

TABLE 2. Localization of the aerobactin and enterochelin siderophores in *E. coli* A36-749 during growth under low-iron conditions[a]

Siderophore and growth time (h)[b]	Extracellular activity		Periplasmic activity		Cytoplasmic activity	
	DP	TF	DP	TF	DP	TF
Aerobactin						
1	−	−	−	−	−	−
2	+	−	−	−	−	−
3	+	+	+	−	−	−
4	+	+	+	+	+	−
5–8	+	+	+	+	+	+
Enterochelin						
1	−	−	−	−	−	−
2	−	−	+	−	−	−
3	−	−	+	+	+	−
4	−	−	+	+	+	−
5	−	−	+	+	+	−
6	−	−	+	−	+	+
7	−	−	+	−	+	+
8	−	+	+	+	+	+

[a] Strain A36-749 (10^7 cells per ml) was grown aerobically for 8 h at 37°C in M9-CA medium containing either 200 μM 2,2′-dipyridyl (DP) or 3.12 μM transferrin (TF).

[b] Localization and activity of aerobactin and enterochelin were estimated as described in Materials and Methods; + and − indicate activity and no activity, respectively.

lin in delivering iron from transferrin to bacterial cells in vitro. The advantage that aerobactin has over enterochelin for growth in transferrin might be explained by (i) lack of activity of enterochelin, (ii) antagonism between the two siderophores, (iii) genetic preferential induction of aerobactin, as presumed by Williams and Carbonetti (28), (iv) preferential excretion of aerobactin, or (v) a more efficient cell-bound aerobactin-dependent mechanism, as suggested by Stuart et al. (25). The latter possibility was not consistent with our results, since no contact between the aerobactin receptor and transferrin molecules was required for aerobactin-mediated removal of transferrin-bound iron. In contrast to mutants defective in the synthesis of both siderophores, aerobactin-deficient mutants capable of producing enterochelin overcame the bacteriostatic effect of transferrin with time. This demonstrated that enterochelin was able to remove transferrin-bound iron. Strains with or without a functional aerobactin system excreted enterochelin at the same time during growth in transferrin. This indicated that the enterochelin-dependent systems in these two types of strains were expressed similarly and that there was no antagonism between the two siderophores. Although enterochelin was belatedly secreted, it accumulated early in the bacterial periplasm. The growth time at which aerobactin was first evidenced was also that at which enterochelin was first detected in the cell periplasmic compartment. This strongly suggests simultaneous derepression of the aerobactin and enterochelin genes rather than differential induction of the two systems. Genetic evidence for a common regulatory mechanism in several iron-related genes (4, 16) strengthens this presumption. Whatever the chelator used to restrict the availability of iron, aerobactin was much more rapidly secreted than enterochelin. We propose that the disadvantage of enterochelin with respect to aerobactin in competing with transferrin for iron was merely due to the extensive retardation of its excretion with respect to aerobactin and thus was a result of the provisional deficiency of its functional cognate uptake system.

In comparison with transferrin, 2,2′-dipyridyl induced greater effective involvement of enterochelin, probably by a more permanent function as iron-binding compounds in the bacterial envelope. Indeed, although enterochelin was synthesized early and was able alone to supply sufficient iron to cells growing exponentially in 2,2′-dipyridyl, its release occurred only during stationary growth. Moreover, excreted-enterochelin production was not caused by an increase of the external 2,2′-dipyridyl concentration. All of these facts suggest that enterochelin competed with 2,2′-dipyridyl for iron in the intracellular compartment. Enterochelin excretion during stationary bacterial growth would result from a nonspecific mechanism such as release of periplasmic contents. Differences in the effects of transferrin and 2,2′-dipyridyl on enterochelin efficiency may be explained by the molecular masses and iron chelation properties of these compounds. Transferrin is a specific Fe^{3+}-chelating hydrophilic protein confined to the extracellular space. In contrast, 2,2′-dipyridyl is a strong Fe^{2+}-complexing hydrophobic agent sufficiently small to settle in cell membranes and enter into bacterial cells (8). We believe that the full efficiency of the enterochelin-mediated system in response to severity of iron stress depends on the cellular location of the effective chelating action of the agents causing iron deprivation. It is likely that data obtained from bacteria grown in medium containing transferrin most closely describe in vivo conditions.

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